A DISSERTATION FOR THE DEGREE OF MASTER OF SCIENCE

Characterization of *Campylobacter jejuni* isolates from dairy cattle farms in Korea

한국의 젖소 농장에서 캠필로박터제주니 분리주의 특성분석

August 2015

Hung Wui Ho

Department of Veterinary Medicine

College of Veterinary Medicine

The Graduate School

Seoul National University

Characterization of *Campylobacter jejuni* isolates from dairy cattle farms in Korea

Supervisor: Seongbeom Cho, D.V.M., Ph. D.

A dissertation submitted in partial fulfillment of the requirement for the

Degree of Master of Science

April 2015

Seoul National University

The Graduate School

Department of Veterinary Medicine

Hung Wui Ho

Approved,

June 2015

Chairman Han Sang Yoo (Signature)

Vice-chairman Seongbeom Cho (Signature)

Committee member Doug-Young Ryu (Signature)

ABSTRACT

Characterization of *Campylobacter jejuni* isolates from dairy cattle farms in Korea

Hung Wui Ho

Department of Veterinary Medicine

College of Veterinary Medicine

The Graduate School

Seoul National University

Campylobacter jejuni is a major foodborne pathogen that is being increasingly identified in various countries worldwide. In this study, 298 samples from 15 dairy cattle farms in Korea were collected between August 2012 and September 2013 in order to measure the prevalence of *C. jejuni*. These cattle isolates, together with *C. jejuni* from other sources (chicken, human, and duck), were characterized based on virulence and survival gene profiles, pulsed-field gel

electrophoresis (PFGE) analysis, and *flaA* typing. A total of 71 *C. jejuni* strains were isolated from the 298 samples, and *C. jejuni* was found to be prevalent in 9 of the 15 cattle farms. *HtrA* was expressed in all 111 isolates tested, whereas 4 other genes (*flhB*, *cdtB*, *perR*, *crsA*) were present in most isolates; by contrast, *virB11* and *hcp* were detected in 12 (10.8%) and 54 (48.6%) of the 111 isolates, respectively. Based on a 90%-similarity criterion, 46 PFGE patterns, 39 *flaA*patterns, and 60 composite patterns were detected from the PFGE analysis and *flaA* typing results. The discriminatory indices for PFGE, *flaA* typing, and composite results were 0.9584, 0.9445, and 0.9766, respectively. Similar band patterns were observed between cattle farms and between cattle, chicken, and human isolates. In conclusion, diverse *C. jejuni* genotypes were identified in cattle farms in Korea, and the tested isolates possessed virulence and survival-factor genes that might present a risk for human campylobacteriosis. Therefore, strict hygiene measures must be developed in cattle farms in order to reduce the threat of human campylobacteriosis to the public.

Keywords:*Campylobacter jejuni*, prevalence, dairy cattle farms, virulence and survival-factor genes, PFGE, *flaA* typing

Student Number: 2013-23933

CONTENTS

LIST OF ABBREVIATIONS

CDC	Centers for Disease Control and Prevention
CdtB	Cytolethal distending toxin B
CrsA	Surface-layer (S-layer) protein gene
DI	Discriminatory index
EU	European Union
*FlaA*typing	Flagellin gene A typing
FlhB	Flagellar biosynthetic protein gene
Hcp	Hemolysin co-regulated protein gene
FMD	Foot-and-mouth disease
GBS	Guillain- Barrésyndrome
HtrA	High-temperature requirement gene
MLST	Multilocus sequence typing

PCR	Polymerase chain reaction
PerR	Peroxidase stress regulator gene
PFGE	Pulsed-field gel electrophoresis
QIA	Animal and Plant Quarantine Agency, Korea
RADP	Polymorphic DNA typing
ST	Sequence type
T3SS	Type III secretion system
T4SS	Type IV secretion system
T6SS	Type VI secretion system
VirB11	Type IV secretion system protein *VirB11* gene

INTRODUCTION

Campylobacter spp. are common foodborne pathogens that cause human enteritis in several industrialized countries (Tauxe et al., 1992). Among *Campylobacter* spp., *Campylobacter jejuni* accounts for 80%–90% of human infections in most parts of the world (Skirrow, 1990). In severe cases of *C. jejuni* infection, certain patients can develop serious immunoreactive complications such as Guillain-Barré syndrome and Miller Fisher syndrome (Kuroki et al., 1993). In South Korea, as in other countries, campylobacteriosis outbreak cases and patients numbers have recently been increasing (Korea Ministry of Food and Drug Safety, 2015).

Poultry was reported to be one of the main sources of human campylobacteriosis (Stanley and Jones, 2003), and another attribution study suggested that the second highest number of human *C. jejuni* infections originated from ruminants (Mughini Gras et al., 2012). Whereas the consumption of undercooked chicken is associated with sporadic cases, contaminated milk has been identified as a source of large-scale outbreaks of campylobacteriosis (CDC, 2009). Furthermore, infected ruminants in farms might continue to shed bacteria, which could increase the infection in other animals or humans by contaminating the environment (Quinn, 1998).

The release of the complete genome of *C. jejuni* NCTC 11168 in 2000 allowed a deep insights into some of the genetic determinants responsible for *C. jejuni* pathogenesis, such as the determinants for motility, chemotaxis, adhesion, invasion, and production of toxins that affect the host (Bolton, 2015). Moreover, the structure of *Campylobacter* features a few secretion systems that play a role in virulence, such as type III (T3SS), type IV (T4SS), and recently identified type VI (T6SS) secretion systems (encoded by *flhB*, *virB11*, and *hcp*), which transport protein toxins from the bacterial cytoplasm into the host or transport extracellular factors (Koolman et al., 2015). Although *Campylobacter* are fastidious organisms that require microaerophilic and thermophilic conditions to survive, they are primarily foodborne pathogens (Bolton, 2015). Therefore, certain survival-factor genes such as *crsA*, *katA*, *sodB*, *htrA*, and *perR* have been proposed to enable *Campylobacter* to respond to the stresses encountered along the food-production chain (Bolton, 2015; Kim et al., 2015; Koolman et al., 2015).

The subtyping of isolates from distinct sources generates epidemiological linkage information that might be useful for assessing future infection risk, controlling disease, and determining the source of *Campylobacter* infection (Han et al., 2007). Among the subtyping methods available, pulsed-field gel electrophoresis (PFGE), which is highly discriminatory, is a widely recognized standard technique used by PulseNet to subtype *Campylobacter*, and *flaA* typing is regarded as an easy, rapid, and commonly used genotypic method to

discriminate *Campylobacter* isolates (Nielsen et al., 2000). Because *Campylobacter* might frequently undergo recombination, the use of multiple genotyping methods (composite analysis) could improve species discrimination (Wassenaar and Newell, 2000).

In this study, the objectives were to (a) determine the prevalence of *C. jejuni* among dairy cattle farms in Korea and observe possible farm-management variables that affect the pathogen's farm-level prevalence; (b) characterize the putative virulence and survival-factor genes among *C. jejuni* isolates from cattle and other sources, including chickens, ducks, and humans, by using PCR methods; and (c) determine the genetic relationships of *Campylobacter* from different sources by using PFGE, *flaA* typing, and both PFGE and *flaA* typing (composite analysis).

MATERIALS AND METHODS

Bacterial strains

C. jejuni ATCC 33560 was used as the positive control for PCR and standard culture assays. A total of 53 *C. jejuni* isolates from different sources (39 isolates from chickens, 7 from humans, 5 from ducks, and 2 from cattle slaughterhouses) were kindly provided by Animal and Plant Quarantine Agency, Korea (QIA), and Konkuk University, and these isolates were included in the identification of virulence and survival-factor genes, PFGE analysis, and *flaA* typing.

Cattle farm samples

Samples from 15 dairy cattle farms located in Gyeonggi-do, Korea, were collected between August 2012 and September 2013. All farms were visited once or twice, whereas farm S was visited 4 times (Table 1).

During each visit, stool samples were collected using rectal palpation gloves directly from the rectum of cows in the lactating herds. At least one environmental sample from the lactating herd was collected during each visit. These samples were transported in an ice box to the Laboratory of Veterinary Public Health, SNU, and processed on the same day. Moreover, the farm-management variables of each farm were recorded, including the density of the

lactating herd (normal and high animal density, based on observation), hygiene level of sawdust (Low; very wet, dirty, and slippery, Mid; normal, High; very dry) (Reneau et al., 2005), stamping-out policy due to foot-and-mouth disease (FMD) in 2010–2011, and the main feed used.

Isolation and identification of *C. jejuni*

When the farm samples reached the laboratory, 1 g or 1 mL of each sample was placed in a Whirl-Pak® filter bag (Nasco, USA) and then 9 mL of Bolton broth containing 5% hemolyzed horse blood and a selective supplement (Oxoid, UK) was added. The samples in the filter bags were homogenized and incubated at 42°C overnight microaerobically. On the following day, one loop of the broth was streaked onto modified charcoal cefoperazone deoxycholate agar (mCCDA) containing the supplement (Oxoid, UK), and then the samples were incubated at 42°C for 48 h microaerobically. Next, 2–6 suspected colonies from the mCCDA plates were picked, subcultured in blood agar, and incubated microaerobically at 42°C for 48 h. To identify *C. jejuni* isolates, multiplex PCR targeting the 16S rRNA gene and *cj0414* was performed using the DNA template, according to methods described previously (Yamazaki-Matsune et al., 2007) (Table 2).

DNA extraction

The DNA template was prepared using a simple boiling method. Briefly, isolates from blood agar were suspended in 200 μL of distilled water and boiled for 10 min, iced for 3 min, and centrifuged at 13000 × *g* for 3 min. The supernatant was used for the identification of *C. jejuni*, detection of virulence and survival-factor genes, and *flaA* typing.

Detection of virulence and survival-factor genes

The genes investigated among various sources were the 3 genes encoding types III, IV, and VI secretion systems of *Campylobacter* (*flhB*, *virB11*, and *hcp*, respectively); *cdtB*, the only toxin gene identified in *Campylobacter*; and the survival genes responsible for biofilm formation (*crsA*), oxidative-stress resistance (*perR*), and heat tolerance (*htrA*) (González-Hein et al., 2013; Kim et al., 2015) (Table 2). The consensus sequence was obtained by aligning 4 *perR* DNA sequences from the *C. jejuni* strains NCTC 11168, F2C10, F2E3, and F2E1 by using CLUSTALW in MegAlign program (DNASTAR, USA). Primer-BLAST software was used for designing the *perR* primers based on the consensus sequence. The PCR mixture (final volume, 20 μL) contained 1× EmeraldAmp GT PCR Master Mix (Takara, Japan), 0.25 mM each of the forward and reverse primers, and distilled water. Positive and negative controls used for the PCR assay were *C. jejuni* reference strain ATCC 33560 and nuclease-free distilled water, respectively. The PCR conditions are summarized

in Table 2, and the PCR products were electrophoresed and visualized on 1% agarose gels.

Pulsed-field gel electrophoresis

PFGE procedures were based on CDC's PulseNet protocol and were performed using the restriction enzyme *Sma*I (Takara, Japan). Briefly, *C. jejuni* isolates from trypticase soy agar containing 5% defibrinated sheep blood (TSA-SB) were harvested using a cotton swab and suspended into 2 mL of 0.85% NaCl in a polystyrene round-bottom tube. The suspension was adjusted to 4 on the McFarland scale by using a DensiCHEK plus instrument (bioMérieux, USA), and 200 μL of the adjusted cell suspension was pipetted into 1.5-mL microcentrifuge tubes, after which 10 μL of Proteinase K (20 mg/mL; Invitrogen, USA) was added to each tube. Next, 200 μL of melted 1% pulsed-field certified agarose (Bio-Rad, USA) in TE buffer (10 mM Tris, 1 mM EDTA) was gently suspended in the mixture and dispensed immediately into reusable plug molds. Plugs that solidified were transferred into 50-mL conical tubes containing 25 μL of Proteinase K solution (20 mg/mL) and 5 mL of lysis buffer (50 mM Tris, 50 mM EDTA, pH 8.0, 1% sarcosyl). The tubes were shaken in a water bath for 2 h at 150 rpm, after which each plug was washed in 5 mL of distilled water 2 times for 20 min and then washed 4 more times similarly in TE buffer. Three slices were cut from the plug and incubated at 25°C for 2-h digestion with 20 U of

*Sma*I. The slices were loaded onto cassettes containing 1% pulse-field certified agarose gel in 0.5× TBE buffer (Biosesang, Korea); subsequently, solidified gels were electrophoresed using a Bio-Rad CHEF Mapper in 0.5× TBE at 14°C, with initial and final switch times of 6.76 and 35.38 s, respectively. After electrophoresis for 18 h, the gels were stained with ethidium bromide (10 μg/mL) for 30 min and destained with distilled water twice for 30 min each. Destained gels were viewed under a Gel Doc XR (Bio-Rad, USA). *Salmonella* Braenderup ATCC BAA664 was used as the size ladder marker.

FlaA typing

To perform *fla*A typing, *flaA*, a gene that is approximately 1.7 kb in size, was amplified and digested with the restriction enzyme *Dde*I (New England BioLabs, USA); the primers used here were adopted from previous studies (El-Adawy et al., 2013; Nachamkin et al., 1996) (Table 2). The 25-μL PCR assay mixture was prepared using 1× PCR buffer, 2 mM $MgCl_2$, 200 μM deoxynucleoside triphosphate (dNTP), 0.2 μM each of forward and reverse primers, AmpliTaq Gold® (1.5 U), 1 μL of DNA template, and distilled water. The PCR products were electrophoresed to check for the presence of 1.7-kb *flaA*; if *flaA* was not amplified, PCR was performed again using a wobble reverse primer and/or the DNA template extracted using a HiGene™ Genomic DNA Prep Kit (BioFact, Korea). After confirming the size of the 1.7-kb band, 8 μL of

each PCR product was digested with 5 U of *Dde*I in a 20-μL mixture and then electrophoresed in a 2% agarose gel at 100 V for 2 h. Gels were viewed under a Gel Doc XR. The size-standard marker used with the digested products was a 1 kb Plus DNA Ladder (BioFact, Korea).

Data analysis

The farm-level prevalence of *C. jejuni* was analyzed using Fisher's exact test to identify the farm-management variables that might affect the presence of *C. jejuni* in farms. Fischer's exact test was also used for analyzing differences in the prevalence of virulence and survival-factor genes between sources.

Band profiles of PFGE and *flaA* typing were analyzed using BioNumerics software version 6.6 (Applied Maths, USA). Dendrograms of PFGE and *flaA* typing were generated based on the Dice similarity coefficient with Unweighted Pair Group Method with Arithmetic Mean (UPGMA) by using tolerance windows of 1.5% and 2%, respectively. Isolates that exhibited ≥90% similarity were assigned to the same genotypic pattern. Composite results were calculated using averages from both PFGE and *flaA* typing and with corrected internal weights. The discriminatory index (DI) of PFGE, *flaA* typing, and composite results was calculated using the formula of the Hunter-Gaston index (Hunter and Gaston, 1988).

RESULTS

In this study, the following 298 samples were collected from 15 dairy cattle farms: 266 samples of cattle feces, 29 bedding samples, 1 sample of raw milk, 1 water sample, and 1 forage sample. On a single visit, 4–36 fecal samples and 1–2 bedding samples were collected. The studied farms contained 30–250 cattle (average: 86).

Prevalence of *C. jejuni*

The farm prevalence of *C. jejuni* was 60.0% (9/15) and the individual prevalence was 25.6% (68/266). Among the 32 environmental samples collected from the farms, 3 bedding samples (9.4%) were positive for *C. jejuni*. Table 1 shows the total prevalence of *C. jejuni* in each farm. The statistical test results obtained for the 15 farms showed that high-versus-normal animal density in farms and sawdust's low-to-mid hygiene level compared to high hygiene level ($P = 0.18$ and $P = 0.08$, respectively, one-tailed Fisher's exact test) appeared to affect the presence of *C. jejuni* in farms, although these did not reach statistical significance.

Prevalence of virulence and survival-factor genes

Table 3 summarizes the PCR results obtained for the 7 virulence and survival-factor genes in 111 *C. jejuni* isolates (60 from cattle, 39 from chickens,

7 from humans, and 5 from ducks). *HtrA* was present in all isolates tested, and 4 other genes (*flhB*, *cdtB*, *perR*, *crsA*) were present in most isolates; by contrast, *virB11* and *hcp* were detected in 12/111 (10.8%) and 54/111 (48.6%) isolates, respectively. The results of Fisher's exact test showed that *virB11* and *hcp* from cattle and from other sources differed significantly ($P < 0.05$).

PFGE

Next, 58 *C. jejuni* isolates from 8 of the "positive" farms and 53 isolates from other sources were typed using PFGE and *flaA* typing. All 111 analyzed isolates could be typed using PFGE, and for each isolate, 6–9 restriction fragments were produced (Figure 1). The PFGE dendrogram produced 46 different genotypes; 17 clusters containing >1 isolate in each genotype were generated, and 63% of the genotypes (29/46) were unique (1 isolate/genotype). The number of samples of each allocated genotype ranged from 1 to 13. Six clusters contained isolates originating from different cattle farms, with a maximum of 5 farm isolates exhibiting a similar PFGE pattern. Among the 17 clusters, 1 cluster contained isolates from 3 sources (cattle, chicken, and human), 3 contained isolates from 2 sources, and the remaining 13 contained isolates from a single source.

FlaA typing

Among the 111 isolates analyzed using *flaA* typing, 4 isolates were untypeable. The remaining 107 *C. jejuni* isolates produced 5–9 restriction fragments each (Figure 2). The *flaA* typing generated 39 genotypes and 19 clusters, and 22 of the genotypes were unique. The sample number per genotype ranged from 1 to 18. Five clusters included isolates from distinct cattle farms, with a maximum of 3 farms being allocated to the same *flaA* type. Among the 19 clusters, 1 cluster contained isolates from 3 sources (cattle, chicken, and human), 7 contained isolates from 2 sources, and 11 contained isolates from a single source. *FlaA* type 3 contained isolates from duck, which were shown to feature a distinct PFGE pulsotype.

Composite results

By combining the results of PFGE and *flaA* typing, 60 composite patterns were generated based on a 90%-similarity criterion (Figure 3). Twenty one clusters were formed with the remaining 39 composite types were unique. Each cluster contained 2–11 isolates, with the frequency being highest for Cluster 14 (9.9%), and 72 of the 111 isolates were part of the clusters. Similar patterns between cattle farms were observed in Clusters 8, 11, 12, 14, and 19. Source-wise, Cluster 14 contained isolates from cattle, chicken, and human, whereas Clusters 8, 16, and 20 contained isolates from 2 sources, and the remaining 17

clusters contained isolates from a single source. Meanwhile, Clusters 4, 13, and 16 included isolates originating from different countries. Out of the 21 clusters, only 7 clusters (16 isolates) of the same fingerprinting types contained isolates featuring the same profile of virulence and survival-factor genes.

Discriminatory index

The DI values of PFGE, *flaA* typing, and composite results for the 111 isolates from different sources were 0.9584, 0.9445, and 0.9766, respectively. When only the 58 *C. jejuni* isolates from cattle farms were evaluated, PFGE had a lower DI compared to *flaA* typing (0.8808 vs. 0.9240).

DISCUSSION

The prevalence of *Campylobacter* spp. isolated from retail meat (chicken, pork, beef) in Korea has been comprehensively described in previous studies (Han et al., 2007; Hong et al., 2007), but information regarding the prevalence of *Campylobacter* spp. in cattle farms in Korea remains limited. Because *C. jejuni* can be introduced to humans along the food-processing chain, understanding the farm-level prevalence of *Campylobacter* might facilitate campylobacteriosis control and prevention. The average *C. jejuni* farm prevalence measured here was 60.0%, which differs from the prevalence in other temperate countries such as Canada (6.5%) (Guevremont et al., 2014) but is similar to that in English and Welsh farms (62.5%) (Ellis-Iversen et al., 2009). The prevalence differences observed in other studies might have resulted from the study size, sampling design, and seasonality (Stanley et al., 1998). Here, seasonality is not discussed because most of the samples in this study were collected during summer, and the periodicity in prevalence from one year to the next was not investigated because the study period was short.

The results of Fisher's exact test suggested that a high density of animals in a farm and low-to-mid hygiene level of sawdust affect the presence of *C. jejuni* ($P < 0.2$). These results are similar to those of a study in which an increase of stocking density in ruminant farms was associated with increased prevalence

of *C. jejuni* in fecal-pat samples (Odd Ratio 1.29) (Grove-White et al., 2010). Because *Campylobacter* spp. are sensitive to desiccation (Fernandez et al., 1985), low-hygiene sawdust containing a high moisture level in the litter would be conducive to *Campylobacter* growth (Eberle et al., 2013). These suboptimal housing conditions might weaken the immune system of the animals, which, in turn, would increase the ease with which the animals might be infected with *Campylobacter*. Once *C. jejuni* is established in a farm, high transmission rates of the bacterium among the housed animals and a high excretion rate might also be caused by stress (Grove-White et al., 2010).

In the second part of this study, *C. jejuni* isolates were genetically characterized by detecting virulence and survival-factor genes and by performing PFGE analysis and *flaA* typing. The results obtained were similar to those of other studies, where *flhB*, *cdtB*, *crsA*, and *htrA* were detected in most of the isolates tested (Bang et al., 2003; Datta et al., 2003). An examination of the literature suggests that this is the first report on the detection of *perR* from various sources. Although mutation of *virB11* is recognized to be capable of causing a 6-fold reduction in bacterial adherence (Bacon et al., 2000), the low prevalence of *virB11* measured in this and a previous study (Koolman et al., 2015) suggests that *virB11* might not be necessary for colonization. *Hcp*, which encodes the T6SS, was recently identified to play roles in virulence by influencing cell adhesion, cytotoxicity toward RBCs, and colonization of mice

(Harrison et al., 2014). Because *hcp*-positive *C. jejuni* was detected in patients who experienced increased levels of bloody diarrhea (Harrison et al., 2014), the mid-level prevalence (60%) of *hcp* in the cattle isolates studied here highlights the public health threat posed by *C. jejuni* originating from cattle. However, only one human isolate (20%) was *hcp* positive; this might be due to the small sample size of human isolates, or because human campylobacteriosis in Korea might arise from additional sources that cause infections that lack serious clinical manifestation. However, these data must be supported with the phenotypic characteristics of the isolates before conclusions can be drawn.

In this study, 2 subtyping methods were used to subtype 111 isolates from various sources: PFGE and *flaA* typing. The typeability was higher in the case of PFGE than in the case of *flaA* typing, because 4 of the isolates could not be typed by means of *flaA* typing. Genotype heterogeneity of *C. jejuni* was observed in most of the cattle farms studied, which indicates that multiple sources might have been responsible for the infection. The high diversity of *Campylobacter* in the cattle populations also highlights the importance of diverse transmission to humans. Isolates from farm S were grouped into the same composite type (Cluster 6; Figure 3) even though they were obtained at different times during the study. This result underscores the persistence of *Campylobacter* that might continue to pose a threat to the public. However, because the samples in this study were not collected periodically, it is not known whether bacteria of a given

genotype continue to colonize the animals in a farm, or are lost from a farm and then re-infect the animals.

The composite results showed that certain genotype patterns were present that were indistinguishable between cattle farms in Gyeonggi-do. Similar genotype patterns between different sources (cattle, human, and chicken) were also demonstrated. Although a direct link between farms and sources was not studied, transmission between them could be considered to occur. Because *Campylobacter* is present in contaminated water sources or soil (Blaser, 1997), this environmental reservoir might also act as a point source for transmission. A few of the clusters in the composite results included isolates from Korea and from imported chicken meat, which suggests that *C. jejuni* from imported meats might also be a source of infection. In this study, a poor linkage was typically observed between composite types and virulence profiles of the isolates. This is similar to the result of van Deun et al. (2007), who reported that isolates from poultry and humans displayed a weak correlation between their PFGE patterns and virulence profiles.

The DI in this study was similar to that in other studies, in which the DI of PFGE was higher than that of *flaA* typing (Han et al., 2007; Nielsen et al., 2000). However, when only the cattle farm samples were included for calculation, the *flaA*-typing DI was higher than the PFGE DI. This can be explained by the finding that *flaA* exhibits higher inherent genetic instability as compared to the

entire genome of *Campylobacter*, which is relatively stable (Wassenaar et al., 2000). When isolates from various sources were included, the DI of PFGE increased, because *flaA* exhibits limited recombination diversity. This study suggests that *flaA* typing must not be used by itself when evaluating the epidemiological linkage between *C. jejuni* isolates. However, *flaA* typing might be useful when evaluating an outbreak cases that are temporally and spatially related.

In conclusion, diverse *C. jejuni* genotypes were identified in cattle farms in Korea, and the isolates examined possessed genes encoding virulence and survival factors that might present a risk for human campylobacteriosis. Therefore, strict hygiene measures must be developed in cattle farms in order to reduce the threat of human campylobacteriosis to the public. Composite analysis performed using PFGE and *flaA* typing was useful for studying the *C. jejuni* epidemiological linkage information. Systematic sampling and analysis of the phenotypic characteristics of *C. jejuni* are expected in future studies to provide additional information on the genetic relatedness and pathogenicity of isolates from distinct sources.

REFERENCES

Bacon, D.J., Alm, R.A., Burr, D.H., Hu, L., Kopecko, D.J., Ewing, C.P., Trust, T.J., Guerry, P., 2000. Involvement of a plasmid in virulence of *Campylobacter jejuni* 81-176. Infect. Immun. 68, 4384-4390.

Bang, D.D., Nielsen, E.M., Scheutz, F., Pedersen, K., Handberg, K., Madsen, M., 2003. PCR detection of seven virulence and toxin genes of *Campylobacter jejuni* and *Campylobacter coli* isolates from Danish pigs and cattle and cytolethal distending toxin production of the isolates. J. Appl. Microbiol. 94, 1003-1014.

Blaser, M.J., 1997. Epidemiologic and clinical features of *Campylobacter jejuni* infections. J. Infect. Dis. 176 Suppl 2, S103-105.

Bolton, D.J., 2015. *Campylobacter* virulence and survival factors. Food Microbiol. 48, 99-108.

Bui, X.T., Qvortrup, K., Wolff, A., Bang, D.D., Creuzenet, C., 2012. Effect of environmental stress factors on the uptake and survival of *Campylobacter jejuni* in *Acanthamoeba castellanii*. BMC Microbiol. 12, 232.

CDC, 2009. *Campylobacter jejuni* infection associated with unpasteurized milk and cheese--Kansas, 2007. MMWR. Morbidity and mortality weekly report 57, 1377.

Datta, S., Niwa, H., Itoh, K., 2003. Prevalence of 11 pathogenic genes of *Campylobacter jejuni* by PCR in strains isolated from humans, poultry meat and broiler and bovine faeces. J. Med. Microbiol. 52, 345-348.

Eberle, K., Davis, J., Purswell, J., Parker, H., McDaniel, C., Kiess, A., 2013. A one year study of newly constructed broiler houses for the prevalence of *Campylobacter*. Int. J. Poultry Sci. 12, 29-36.

El-Adawy, H., Hotzel, H., Tomaso, H., Neubauer, H., Taboada, E.N., Ehricht, R., Hafez, H.M., 2013. Detection of genetic diversity in *Campylobacter jejuni* isolated from a commercial turkey flock using *flaA* typing, MLST analysis and microarray assay. PLoS One 8, e51582.

Ellis-Iversen, J., Pritchard, G.C., Wooldridge, M., Nielen, M., 2009. Risk factors for *Campylobacter jejuni* and *Campylobacter coli* in young cattle on English and Welsh farms. Prev. Vet. Med. 88, 42-48.

Fernandez, H., Vergara, M., Tapia, F., 1985. Dessication resistance in thermotolerant *Campylobacter* species. Infection 13, 197.

González-Hein, G., Huaracán, B., García, P., Figueroa, G., 2013. Prevalence of virulence genes in strains of *Campylobacter jejuni* isolated from human, bovine and broiler. Braz. J. of Microbiol. 44, 1223-1229.

Grove-White, D.H., Leatherbarrow, A.J., Cripps, P.J., Diggle, P.J., French, N.P., 2010. Temporal and farm-management-associated variation in the faecal-

pat prevalence of *Campylobacter jejuni* in ruminants. Epidemiol. Infect. 138, 549-558.

Guevremont, E., Lamoureux, L., Loubier, C.B., Villeneuve, S., Dubuc, J., 2014. Detection and characterization of *Campylobacter* spp. from 40 dairy cattle herds in Quebec, Canada. Foodborne Pathog. Dis. 11, 388-394.

Han, K., Jang, S.S., Choo, E., Heu, S., Ryu, S., 2007. Prevalence, genetic diversity, and antibiotic resistance patterns of *Campylobacter jejuni* from retail raw chickens in Korea. Int. J. Food Microbiol. 114, 50-59.

Harrison, J.W., Dung, T.T., Siddiqui, F., Korbrisate, S., Bukhari, H., Tra, M.P., Hoang, N.V., Carrique-Mas, J., Bryant, J., Campbell, J.I., Studholme, D.J., Wren, B.W., Baker, S., Titball, R.W., Champion, O.L., 2014. Identification of possible virulence marker from *Campylobacter jejuni* isolates. Emerg. Infect. Dis. 20, 1026-1029.

Hong, J., Kim, J.M., Jung, W.K., Kim, S.H., Bae, W., Koo, H.C., Gil, J., Kim, M., Ser, J., Park, Y.H., 2007. Prevalence and antibiotic resistance of *Campylobacter* spp. isolated from chicken meat, pork, and beef in Korea, from 2001 to 2006. J. Food Protect. 70, 860-866.

Hunter, P.R., Gaston, M.A., 1988. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J. Clin. Microbiol. 26, 2465-2466.

Kim, J.C., Oh, E., Hwang, S., Ryu, S., Jeon, B., 2015. Non-selective regulation of peroxide and superoxide resistance genes by *perR* in *Campylobacter jejuni*. Front Microbiol. 6, 126.

Koolman, L., Whyte, P., Burgess, C., Bolton, D., 2015. Distribution of virulence-associated genes in a selection of campylobacter isolates. Foodborne Pathog. Dis. 12, 424-432.

Korea Ministry of Food and Drug Safety. The statistics for reported foodborne outbreaks in Korea. Available at: http://www.mfds.go.kr/e-stat/index.do, accessed May 15, 2015.

Kuroki, S., Saida, T., Nukina, M., Haruta, T., Yoshioka, M., Kobayashi, Y., Nakanishi, H., 1993. *Campylobacter jejuni* strains from patients with Guillain-Barre syndrome belong mostly to Penner serogroup 19 and contain beta-N-acetylglucosamine residues. Ann. Neurol. 33, 243-247.

Mughini Gras, L., Smid, J.H., Wagenaar, J.A., de Boer, A.G., Havelaar, A.H., Friesema, I.H., French, N.P., Busani, L., van Pelt, W., 2012. Risk factors for campylobacteriosis of chicken, ruminant, and environmental origin: a combined case-control and source attribution analysis. PLoS One 7, e42599.

Nachamkin, I., Ung, H., Patton, C.M., 1996. Analysis of HL and O serotypes of *Campylobacter* strains by the flagellin gene typing system. J. Clin. Microbiol. 34, 277-281.

Nielsen, E.M., Engberg, J., Fussing, V., Petersen, L., Brogren, C.H., On, S.L., 2000. Evaluation of phenotypic and genotypic methods for subtyping *Campylobacter jejuni* isolates from humans, poultry, and cattle. J. Clin. Microbiol. 38, 3800-3810.

Quinn, J., 1998. Clinical strategies for serious infection: a North American perspective. Diagn. Micr. Infect. Dis. 31, 389-395.

Reneau, J.K., Seykora, A.J., Heins, B.J., Endres, M.I., Farnsworth, R.J., Bey, R.F., 2005. Association between hygiene scores and somatic cell scores in dairy cattle. J. Am. Vet. Med. Assoc. 227, 1297-1301.

Skirrow, M., 1990. *Campylobacter*. The Lancet 336, 921-923.

Stanley, K., Jones, K., 2003. Cattle and sheep farms as reservoirs of *Campylobacter*. J. Appl. Microbiol. 94 Suppl, 104s-113s.

Stanley, K.N., Wallace, J.S., Currie, J.E., Diggle, P.J., Jones, K., 1998. The seasonal variation of thermophilic campylobacters in beef cattle, dairy cattle and calves. J. Appl. Microbiol. 85, 472-480.

Tauxe, R.V., 1992. Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations. In: Nachamkin, I., Blaser, M., Tompkins, L., editors. Campylobacter jejuni: current and future trends. Washington: American Society for Microbiology.

van Deun, K., Haesebrouck, F., Heyndrickx, M., Favoreel, H., Dewulf, J., Ceelen, L., Dumez, L., Messens, W., Leleu, S., van Immerseel, F., Ducatelle, R.,

Pasmans, F., 2007. Virulence properties of *Campylobacter jejuni* isolates of poultry and human origin. J. Med. Microbiol. 56, 1284-1289.

Wassenaar, T.M., Fry, B.N., van der Zeijst, B.A., 1995. Variation of the flagellin gene locus of *Campylobacter jejuni* by recombination and horizontal gene transfer. Microbiology 141 (Pt 1), 95-101.

Wassenaar, T.M., Newell, D.G., 2000. Genotyping of *Campylobacter* spp. Appl. Environ. Microbiol. 66, 1-9.

Yamazaki-Matsune, W., Taguchi, M., Seto, K., Kawahara, R., Kawatsu, K., Kumeda, Y., Kitazato, M., Nukina, M., Misawa, N., Tsukamoto, T., 2007. Development of a multiplex PCR assay for identification of *Campylobacter coli*, *Campylobacter fetus*, *Campylobacter hyointestinalis* subsp. *hyointestinalis*, *Campylobacter jejuni*, *Campylobacter lari* and *Campylobacter upsaliensis*. J. Med. Microbiol. 56, 1467-1473.

Table 1. Information on the 15 cattle farms included in this study and their *C. jejuni* prevalence

Farm	Farm size	Density of farm[a]	Hygiene of sawdust[b]	Stamping out due to FMD	Main feed	Total of visit	Year	*C. jejuni* positive isolates/ samples collected (%)
1	250	Normal	High	No	TMR[c]	1	2012	0/8 (0.0)
2	60	Normal	Mid	Yes	TMR	1	2012	2/10 (20.0)
3	60	High	Low	No	TMR	1	2012	2/8 (25.0)
4	50	High	Low	No	Hay	2	2012	2/9 (22.2)
							2013	2/6 (33.3)
5	30	High	Low	No	TMR	1	2012	1/7 (14.3)
6	40	Normal	High	No	TMR	1	2012	0/9 (0.0)
7	160	Normal	High	Yes	TMR	2	2012	7/10 (70.0)
							2013	1/8 (12.5)
8	150	Normal	High	Yes	TMR	1	2012	0/10 (0.0)
9	50	Normal	High	No	TMR	1	2012	0/10 (0.0)
10	70	Normal	Low	No	TMR	2	2012	0/10 (0.0)
							2013	0/4 (0.0)
11	30	Normal	Mid	Yes	TMR	2	2012	6/9 (66.7)

Farm	Farm size	Density of farm[a]	Hygiene of sawdust[b]	Stamping out due to FMD	Main feed	Total of visit	Year	*C. jejuni* positive isolates/ samples collected (%)
							2013	5/7 (71.4)
12	50	Normal	Low	Yes	TMR	2	2012	8/10 (80.0)
							2013	2/9 (22.2)
13	80	Normal	High	No	TMR	2	2012	7/11 (63.6)
							2013	3/8 (37.5)
14	60	Normal	High	Yes	TMR	1	2012	0/12 (0.0)
S	150	Normal	High	No	Hay	4	2012	3/24 (12.5)
							2012	0/28 (0.0)
							2013	14/32 (43.6)
							2013	6/36 (16.7)
Total						24		71/298 (23.8)

[a] Density of animals in the lactating herd in each farm was recorded based on observation, and was compared between all farms.

[b] Hygiene level of sawdust: Low; very wet, dirty, and slippery, Mid; normal, High; very dry (Reneau et. al., 2005).

[c] TMR: Total mixed ration.

Table 2. Oligonucleotide primers used in this study

Gene	Primer	Nucleotide sequences (5' to 3')	Amplicon size (bp)	PCR conditions	References
16S rRNA	F	GGATGACACTTTTCGGAGC	816	95°C for 15 min/ 25 cycles of 95°C x 30 s, 58°C x 1 min 30 s and 72°C x 1 min/ 72°C for 7 min[a]	(Yamazaki-Matsune et al., 2007)
	R	CATTGTAGCACGTGTGTC			
cj0414	F	CAAATAAAGTTAGAGGTAGAATGT	161		
	R	CCATAAGCACTAGCTAGCTGAT			
flaA	F	GGATTTCGTATTAACA	~1700	94°C for 10 min/ 35 cycles of 94°C x 1 min, 45°C x 1 min and 72°C x 2 min/ 72°C for 7 min	(El-Adawy et al., 2013; Nachamkin et al., 1996)
	R	CTGTAGTAATCTTAAAACATTTTG			
	R-Wob[b]	CTGTARYAATCTTAAAACATTTTG			(El-Adawy et al., 2013)
flhB	F	TGGCAGGCGAAGATCAAGAA	549	95°C for 1 min/ 35 cycles of 95°C x 1 min, 55°C x 1 min and 72°C x 1 min/ 72°C for 5 min	(Koolman et al., 2015)
	R	GCCAAGTAAGCTGTGCAACC			
virB11	F	TCAGGTGGAACAGGAAGTGG	329	95°C for 1 min/ 35 cycles of 95°C x 1 min, 54°C x 1 min and 72°C x 1 min/ 72°C for 5 min	(Koolman et al., 2015)
	R	GCTTTGATCGCGTCTTCTGG			
hcp	F	CAAGCGGTGCATCTACTGAA	463	95°C for 2 min/ 30 cycles of 95°C x 15 s, 54°C x 30 s and 72°C x 30 s / 72°C for 5 min	(Harrison et al., 2014)
	R	TAAGCTTTGCCCTCTCTCCA			

Gene	Primer	Nucleotide sequences (5' to 3')	Amplicon size (bp)	PCR conditions	References
cdtB	F	GCTCCTACATCAACGCGAGA	376	95°C for 1 min/ 35 cycles of 95°C x 1 min, 55°C x 1 min and 72°C x 1 min/ 72°C for 5 min	(Koolman et al., 2015)
	R	ACTACTCCGCCTTTTACCGC			
perR	F	CCCTTCAATCTCTTTAGCGACG	153	95°C for 4 min/ 30 cycles of 95°C x 30 s, 55°C x 30 s and 72°C x 30 s / 72°C for 7 min	This study
	R	ATACCACCACATTTGGCGCA			
crsA	F	CACAGTCAGTGAAGGTGCTT	878	94°C for 3 min/ 30 cycles of 94°C x 30 s, 58°C x 30 s and 72°C x 30 s/ 72°C for 5 min/ 65°C for 5 min	(González-Hein et al., 2013)
	R	ACTCGCACAATCGCTACTTC			
htrA	F	CCATTGCGATATACCCAAACTT	130	94°C for 5 min/ 30 cycles of 94°C x 15 s, 52°C x 20 s and 72°C x 15 s/ 72°C for 3 min	(Bui et al., 2012)
	R	CTGGTTTCCAAGAGGGTGAT			

[a] Multiplex PCR conditions for 16S rRNA gene and *cj0414*.

[b] *flaA*-R-Wob primer was used as the reverse primer if the first set of primers (*flaA*-F and *flaA*-R) did not amplify *flaA*.

Table 3. Distribution of 7 virulence and survival-factor genes in *Campylobacter jejuni* isolates from different sources

Category	Gene	Source[a]			
		Cattle (n=60)	Chicken (n=39)	Human (n=7)	Duck (n=5)
T3SS[b]	*flhB*	57 (95.0)	39 (100.0)	7 (100.0)	5 (100.0)
T4SS[c]	*virB11*	10 (16.7)	2 (5.1)	0 (0.0)	0 (0.0)
T6SS[d]	*hcp*	36 (60.0)	14 (35.9)	2 (28.6)	2 (40.0)
Cytotoxin	*cdtB*	58 (96.7)	39 (100.0)	7 (100.0)	5 (100.0)
Stress response	*crsA*	60 (100.0)	39 (100.0)	6 (85.7)	5 (100.0)
	perR	59 (98.3)	39 (100.0)	7 (100.0)	5 (100.0)
	htrA	60 (100.0)	39 (100.0)	7 (100.0)	5 (100.0)

[a] Figures in parentheses refer to the percentage of isolates positive for the gene in each category.

[b,c,d] T3SS: Type III secretion system. T4SS: Type IV secretion system. T6SS: Type VI secretion system.

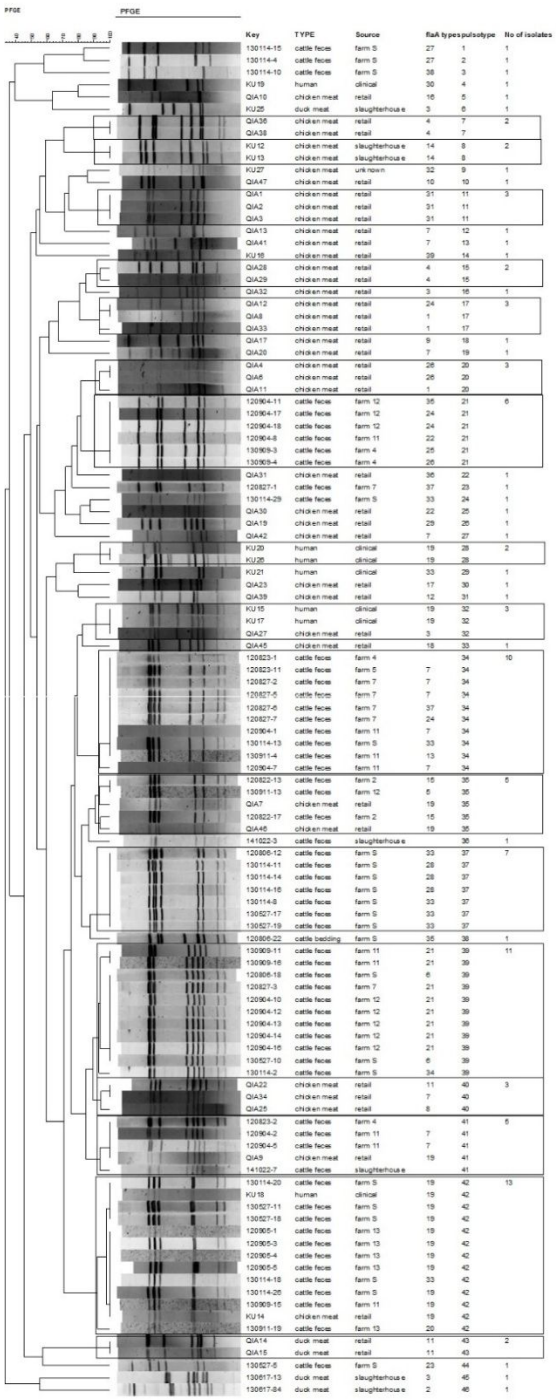

Fig. 1. Dendrogram of 111 *C. jejuni* isolates showing the 46 PFGE types based on a 90%-similarity criterion and a 1.5% tolerance window following digestion with *Sma*I. The box indicates the 90%-similarity threshold. Discriminatory index (DI): 0.9584.

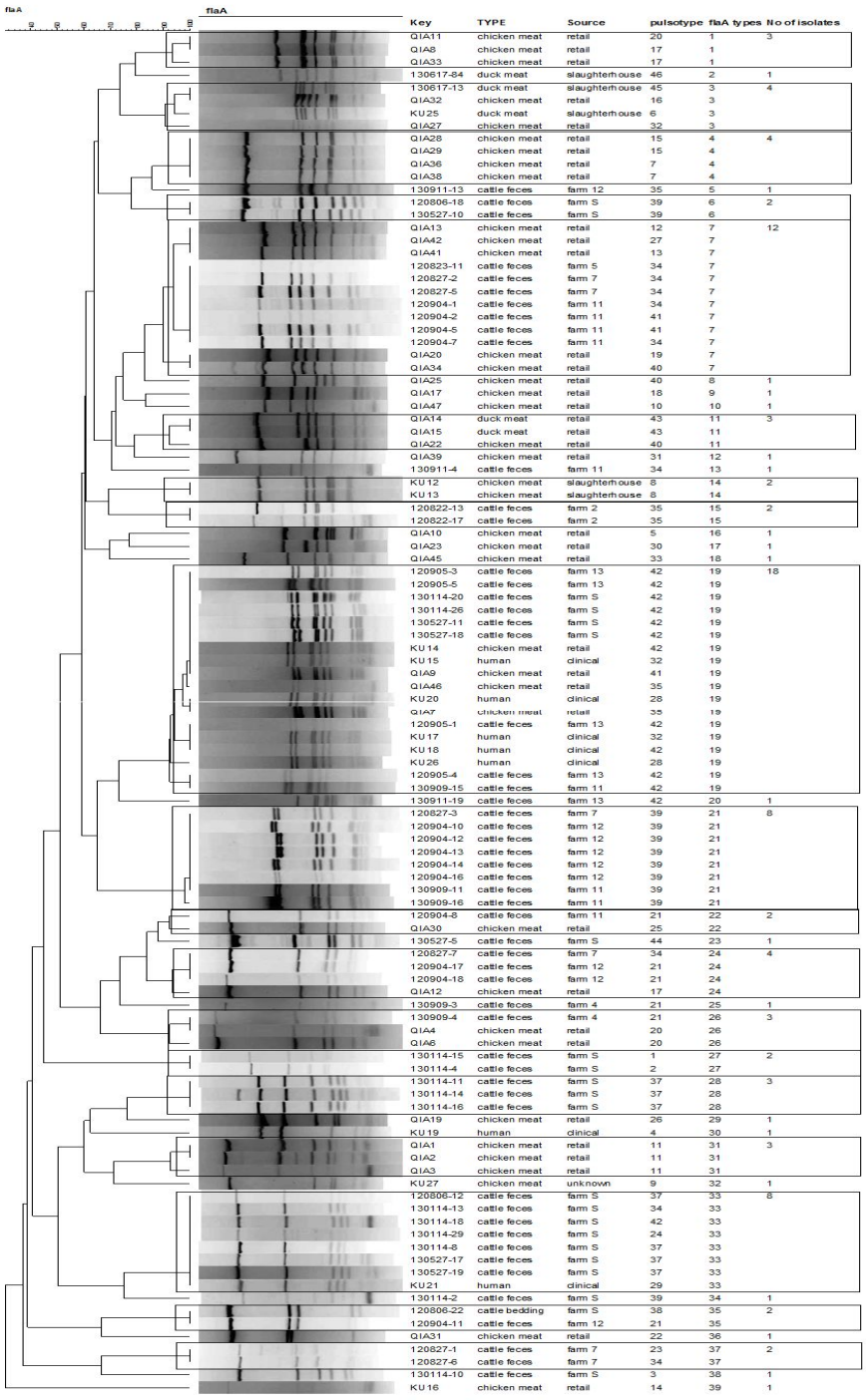

Fig. 2. Dendrogram of 107 *C. jejuni* isolates showing the 39 *flaA* types based on a 90%-similarity criterion and a 2% tolerance window following digestion with *Dde*I. The box indicates the 90%-similarity threshold. Discriminatory index (DI): 0.9445.

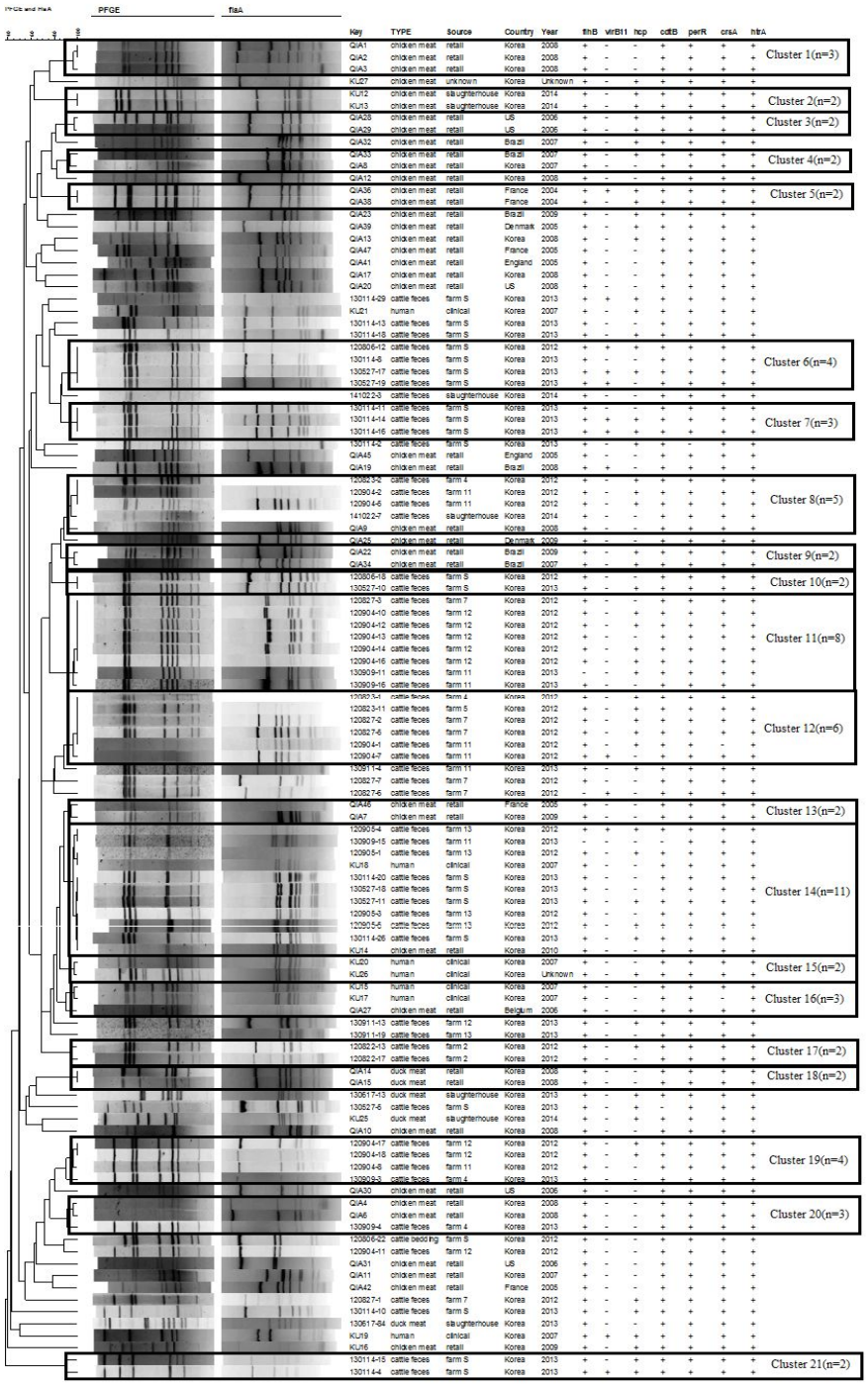

Fig. 3. Dendrogram showing the composite types of 111 *C. jejuni* isolates based on the average similarity matrices of PFGE and *flaA* typing. The box indicates the 90%-similarity threshold. Discriminatory index (DI): 0.9766.

국문초록

한국의 젖소 농장에서 캠필로박터제주니 분리주의 특성 분석

서울대학교 대학원

수의학과수의공중보건전공

호홍위

(지도교수: 조성범)

2012 년 8 월 -2013 년 9 월에 15 개 젖소 농장으로부터 채취한 총 298 개의 샘플에서 분리배양을 기반으로 한 PCR 방법을 통해 *Campylobacter jejuni* 의 유병률을 확인하였다. 총 298 개 샘플 (분변 266, 환경샘플 32) 중 71 개 *C. jejuni* 가 검출되었으며, 농장 단위에서는 15 개 농장중 9 개 농장이 *C.jejuni* 양성이었다. 15 개 농장을 대상으로 한 Fisher 정확검정 (Fisher's exact test)결과 사육의 밀집도가 높거나 톱밥의 위생상태가 낮음에서 중간수준인 경우가(각각 P=0.18 및 P=0.08, one tailed Fisher's exact test) 농장의 *C. jejuni* 존재 가능성에 영향을 줄 수 있는 요인으로 추정되었다. 기 연구에서 분리된 71 개의 분리주 중 58 개와 실험실에서 보유하고 있었던 다양한 유래의 53 개 *C. jejuni* 분리주를 PFGE, *flaA* typing 과 병원성 유전자 확인을 통해 분리주 간의 유전적 연관성 및 병원성을 조사하였다. 실험결과 *htrA* 은 모든

isolates 에서, 111 isolates 중 *virB11* 의 경우 12 개 (10.8%), *hcp* 54 개 (48.6%)로 나타났으며 이 외에 나머지 4 개 genes (*flhB, cdtB, perR, crsA*) 의 경우 대부분의 isolates 가 가지고 있는 것으로 확인되었다. 90% 유사성 기준 (90% similarity criterion) 에 따라 46 개의 PFGE 패턴, 39 개의 *flaA* 패턴 및 60 개의 composite type 을 확인하였다. PFGE, *flaA*, composite type 분석의 차별적지수(Discriminatory index. D index)는 각각 0.9584, 0.9445, 0.9766 였다. Composite 분석 결과는 농장간 그리고 소-사람-닭 유래 분리주간에 유사한 패턴을 보였다. 결론적으로 이번 실험의 결과를 통해 농장내에 다양한 유전형의 *C. jejuni* 가 존재 하며 사람의 campylobacteriosis 는 소에서 유래되었을 가능성이 있음을 확인 하였다. 그러므로 공중보건학적으로 campylobacteriosis 의 위험을 줄이기 위해서는 소 농장에서의 *C. jejuni* 유병률을 낮출 수 있는 엄격한 위생관리 방법이 개발 되어야한다.

Keywords: *Campylobacter jejuni*, prevalence, dairy cattle farms, virulence and survival-factor genes, PFGE, *flaA* typing

Student Number: 2013-23933